Pestic. Sci. 1997, **51**, 33–38

Structural Modifications Increase the Insecticidal Activity of Ryanodine

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(Received 25 September 1996; revised version received 20 January 1997; accepted 1 May 1997)

Abstract: The toxicity of ryanodine (**1**) and 9,21-didehydroryanodine (**2**) (the principal active ingredients of the botanical insecticide ryania) to adult female house flies (*Musca domestica* L.) is attributable to binding to the ryanodine receptor (*ryr*) and thereby disrupting the Ca^{2+}-release channel. These ryanoids, assayed in house flies with piperonyl butoxide (PBO) to suppress cytochrome P450-dependent detoxification, give injected KD_{50} values of 0·07–0·11 $\mu g\ g^{-1}$, injected LD_{50} values of 0·39–0·45 $\mu g\ g^{-1}$ and topical LD_{50} values of 12–50 $\mu g\ g^{-1}$. They inhibit the [^{3}H]ryanodine binding site of house fly and rabbit muscle with IC_{50} values of 3–10 nM. This study examines the effect of structure on potency, with 15 variants of the cyclohexane substituents, two 4,6-cyclic boron and two methylated derivatives, and four modifications of the isopropyl and ester substituents. The most effective compound examined was 10-deoxy-**2** (**3**) which was more potent than **2** by 2–4-fold on injection and 29-fold applied topically following PBO (LD_{50} 0·41 $\mu g\ g^{-1}$). Additional high-potency compounds were 10-oxo-**1** and the cyclohexane variants with lactam, 21-*nor*-9-oxo and 21-*nor*-10-deoxy substituents. Other modifications usually reduced toxicity. The injected knockdown potency of the ester ryanoids was generally related to their effectiveness in competing with [^{3}H]ryanodine at the *ryr* of rabbit skeletal muscle. Two non-ester ryanoids, ryanodol and 9,21-didehydroryanodol, were found to be more toxic than predicted from their potency at the *ryr* and may therefore act in a different manner such as at a K^+ channel, as suggested by Usherwood and Vais. Clearly ryanoids are challenging prototypes for a potential new generation of insecticides.

Pestic Sci., **51**, 33–38, 1997
No. of Figures: 4. No. of Tables: 2. No. of Refs: 13

Key words: Ca^{2+} release channel, didehydroryanodine, K^+ channel, *Musca domestica*, ryanodine, ryanodine receptor, ryanodol, synergism

1 INTRODUCTION

Ryanodine (Fig. 1, **1**) and 9,21-didehydroryanodine (**2**) are the principal active ingredients in the botanical insecticide ryania, the ground stemwood of *Ryania speciosa* Vahl.[1–3] These ryanoids act primarily at the Ca^{2+}-release channel (also referred to as the Ca^{2+}-ryanodine receptor or *ryr*) in both mammals[4] and insects.[5] The *ryr*s of house fly, cockroach, mouse brain and rabbit muscle are similar in sensitivity to **1** and **2** with concentrations for 50% inhibition (IC_{50}) of 3–10 nM.[2,5] Ryanodol and 9,21-didehydroryanodol (the hydrolysis products of **1** and **2**, respectively) are more selective toxicants for insects versus mammals than the corresponding esters **1** and **2**.[2] This may be due to a greater action of the non-ester ryanoids on voltage-

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Contract grant sponsor: National Institute of Environmental Health Sciences; Contract grant number: PO1 ES00049.

 Pestic. Sci. 0031-613X/97/$17.50. Printed in Great Britain

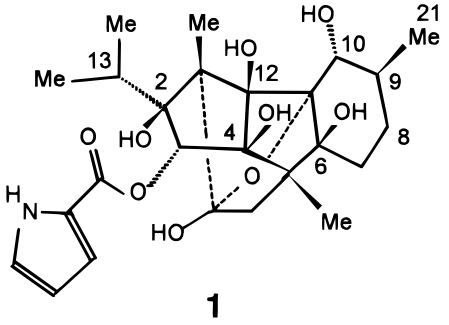

Fig. 1. Structure of ryanodine (**1**).

sensitive K^+ channels and of the ester ryanoids on the Ca^{2+}-release channel.[6,7]

Attempts to improve on the insecticidal activity of **1** and **2** by isolating other natural ryanoids[3] or preparing semi-synthetic analogs[8,9] has had only limited success. These involved in the most part highly-sensitive assays with adult female house flies (*Musca domestica* L.) by determining the knockdown (KD) activity after topical treatment with piperonyl butoxide (PBO) (to minimize cytochrome P450-dependent detoxification) and then injection of the ryanoid. We recently examined 54 new ryanoids and noted considerable structural specificity with variations in the cyclohexane ring and the hydroxyl substituents in their binding to mammalian receptor preparations from skeletal and cardiac muscle and brain compared with their action in a muscle contractility assay.[10,11] The present investigation extends these observations to house flies with the goal of increasing the insecticidal activity, particularly following topical application, by further structural optimization, with emphasis on masking or removing the hydroxyl substituents.

2 MATERIALS AND METHODS

2.1 Chemicals

The ryanoids examined are modified in the cyclohexane substituent (**1**–**15**) (Fig. 2) or are cyclic boron (**16** and **17**) or methylated (**18** and **19**) derivatives or have modifications of the isopropyl and ester substituents (**20**–**23**) (Fig. 3). Sources for or preparation of these compounds are described in our recent reports.[10,11]

2.2 Toxicity to house flies and mice

Adult female house flies (SCR susceptible strain) were individually treated topically with PBO (5 μg) applied as a solution in acetone (0·5 μl) to the ventrum of the

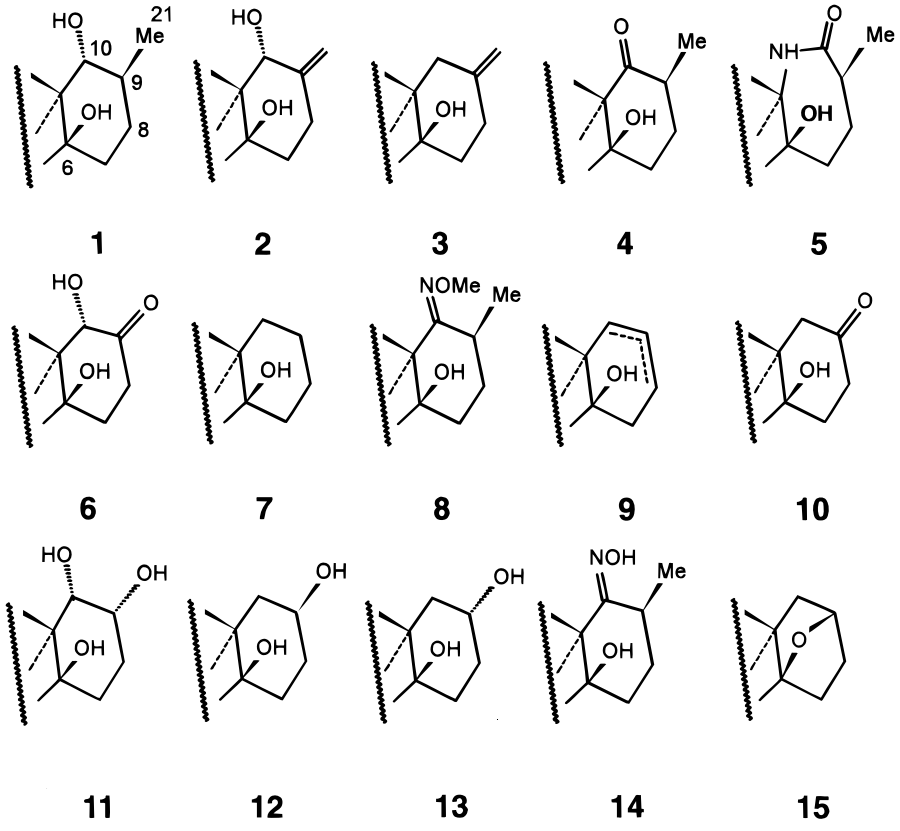

Fig. 2. Partial structures of ryanoids with modifications of cyclohexane substituents (**1**–**15**).

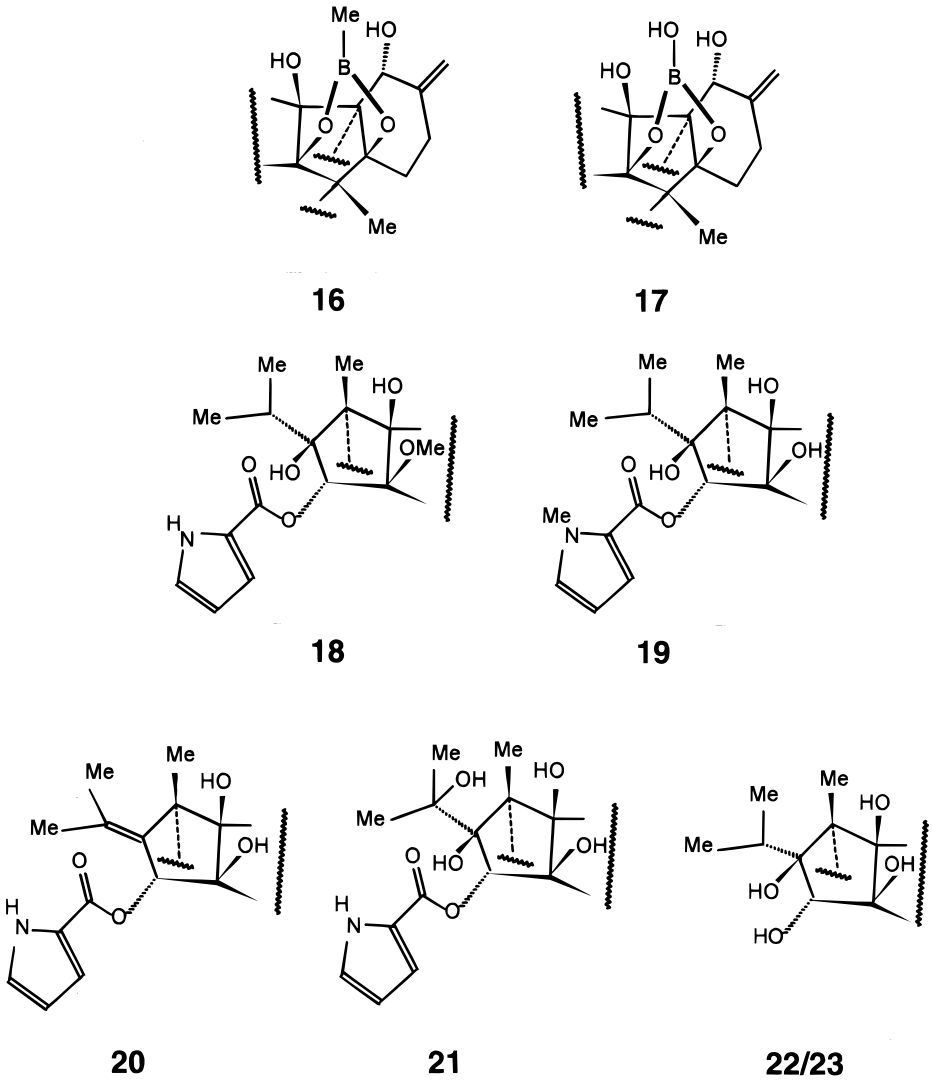

Fig. 3. Partial structures of 4,6-cyclic boron and methylated derivatives (**16**–**19**) and ryanoids with modifications of the isopropyl and ester substituents (**20**–**23**).

abdomen. PBO is known to be a synergist for **1** and **22** in this strain,[2] which contains significant cytochrome P450-dependent detoxifying activity.[12] The test compound was administered 1 h later by intrathoracic injection as a solution in water + ethanol (1 + 1 by volume; 0·22 μl) or by topical application in ethanol (0·5 μl) as above. The knockdown and lethal doses for 50% of the flies (KD_{50} and LD_{50}) are defined on the basis of inability to fly or walk at 4 h and immobility or death at 24 h after administering the test compound. These experiments involved five doses in the toxic range, a dose differential of two- to three-fold, 10 flies for each dose, two or more independent determinations, and log-probit analyses of the cumulative data.

Male albino mice were treated by intraperitoneal (ip) injection with the test compounds in water + ethanol (3 + 1 by volume) and the LD_{50} values were determined at 24 h.

2.3 Potency at ryanodine receptor

Data from our earlier studies[10,11] on ryanoid potency (concentrations for 50% inhibition or IC_{50} values) at the rabbit muscle Ca^{2+}-release channel are used for comparison. Incubation mixtures involved the sarcoplasmic reticulum preparation (20 μg protein) and [^{3}H]ryanodine (1 nM) in pH 7·4 assay buffer (1000 μl) consisting of adenosine triphosphate (5 mM), $CaCl_2$ (400 μM), KCl (0·75 M), sucrose (0·15 M), Hepes (5 mM) and Tris maleate (10 mM). After incubation for 90 min at 37°C, the mixtures were filtered through Whatman GF/C glass fiber filters and washed three times each with ice-cold 150 mM KCl in 10 mM Hepes pH 7·4 (5 ml). Nonspecific binding was determined with 5 μM **2**, resulting in specific binding of >90%. Test ryanoids were introduced in dimethyl sulfoxide (5 ml litre^{-1} final concentration).

3 RESULTS AND DISCUSSION

3.1 Structure–activity relationships for knockdown activity of injected ryanoids (Table 1)

The activities of **1** and **2** are similar and sufficiently high to serve as the basis for examining the effect of structural modifications on potency.

*3.1.1 Modifications of cyclohexane substituents (**1–15**) (Fig. 2)*

The potency of **2** is increased two-fold on removing the 10-hydroxy substituent (**3** versus **2**). There is little, if any, change in activity on conversion to the 10-oxo (**4**), lactam (**5**), 21-*nor*-9-oxo (**6**), and 21-*nor*-10-deoxy (**7**) derivatives and a ~ three-fold potency loss for the methoxime (**8**) and 21-*nor*-10-deoxy-Δ^8/Δ^9 (**9**) compounds. Removal of the C-9(21) methylene group lowers activity four-fold (**3** versus **7**) and introduction of unsaturation into **7** as in **9** reduces KD potency another three-fold. Other variations in structure confer somewhat lower KD activity, including the 21-*nor*-10-deoxy-9-oxo (**10**), 21-*nor*-9_{ax}-hydroxy (**11**), 21-*nor*-10-deoxy-9_{eq}- or 9_{ax}-hydroxy (**12** and **13**), oxime (**14**) and cyclic ether (**15**) derivatives.

*3.1.2 4,6-Cyclic boron (**16** and **17**) and methylated derivatives (**18** and **19**) and modifications of the isopropyl and ester substituents (**20–23**) (Fig. 3)*

The methyl boronate of **2** (**16**) is similar to **2** in potency,

TABLE 1
Relation for Ryanoids Between Injected KD_{50} and LD_{50} for House Flies Pretreated with Piperonyl Butoxide and Relative IC_{50} at the Rabbit Muscle Ca^{2+} Release Channel/Ryanodine Receptor (*ryr*)

		House fly ($\mu g\ g^{-1}$)		
No.	*Compound*	KD_{50}	LD_{50}	*ryr* IC_{50} (*relative*)[a]
Ryania active ingredients				
1	Ryanodine	0·11	0·39	1·0
2	Didehydroryanodine	0·070	0·45	1·0
Modifications of cyclohexane substituents				
3	10-Deoxy-**2**	0·035	0·11	2·7
4	10-Oxo-**1**	0·11	0·26	3·3
5	Lactam	0·13	0·35	2·4
6	21-*nor*-9-Oxo	0·13	3·1	10
7	21-*nor*-10-Deoxy	0·14	0·55	15
8	Methoxime-**1**	0·33	0·65	7·7
9	21-*nor*-10-Deoxy-Δ^8/Δ^9	0·39	1·0	175
10	21-*nor*-10-Deoxy-9-oxo	0·70	2·0	100
11	21-*nor*-9_{ax}-Hydroxy	0·85	14	160
12	21-*nor*-10-Deoxy-9_{eq}-hydroxy	1·0	20	42
13	21-*nor*-10-Deoxy-9_{ax}-hydroxy	1·5	46	500
14	Oxime-**1**	1·7	12	3·8
15	21-*nor*-10-Deoxy-6,9-oxido	6·5	65[b]	630
4,6-Cyclic boron derivatives				
16	Methylboronate-**2**	0·070	0·18	4·5
17	Borate-**2**	0·19	0·61	1·0
Methylated derivatives				
18	4-*O*-Me-**2**	0·36	6·0	3·4
19	*N*-Me-**2**	1·6	25	8·3
Modifications of isopropyl and ester substituents				
20	2-Deoxy-2(13)-dehydro-**2**	0·50	2·7	7·7
21	13-Hydroxy-**1**	1·2	100[b]	50
22	Ryanodol	2·4		3500
23	Didehydroryanodol	1·1	15	1000

[a] IC_{50} values for rabbit skeletal muscle *ryr* are normalized to the value for compound **1** in the same experiment, which varied from 3·5 to 10 nM. Data for compounds **1–21** from Refs 10 and 11 and supporting information cited therein. Results for compounds **22** and **23** from Ref. 2.
[b] Extrapolated from 35–40% mortality at 50 $\mu g\ g^{-1}$.

whereas the borate (**17**) is two- to three-fold less active; these compounds probably act by release of **2** on exchange.[10] Methylation of the 4-hydroxyl of **2** (**18**) reduces activity five-fold and of the pyrrole nitrogen of **2** (**19**) by 23-fold. Similarly, we have shown[9] for a modified skeleton that high toxicity to house flies requires the polar OH or NHOH group at C-4 whereas these groups at C-12 are less important. The isopropyl substituent can be modified with only seven- to 11-fold potency loss by deoxygenation at the 2-position, resulting in a double bond (**20**), or by introducing a 13-hydroxyl substituent (**21**). The ester group is important with 16- to 22-fold potency loss on hydrolysis to ryanodol (**22**) or didehydroryanodol (**23**), which nevertheless retain very significant activity (KD_{50} 1·1–2·4 $\mu g\ g^{-1}$).

3.2 Relationship between injected KD_{50} and LD_{50} (Table 1)

The LD_{50} values at 24 h range from two- to 83-fold higher than the KD_{50} values at 4 h, i.e. many of the house flies suffering knockdown subsequently recover. The compounds of greatest interest are those of lower or higher LD_{50}/KD_{50} ratios, implying lesser or greater ease of metabolism. However, the LD_{50}/KD_{50} ratios are generally related to the potency: compounds with LD_{50} values of 2·7 $\mu g\ g^{-1}$ or below have low ratios of 2·0–6·4; those with LD_{50} values of 3·1 $\mu g\ g^{-1}$ or above have ratios of 7–83. When considered by structure, the latter set of compounds with high ratios have 9-oxo or 9_{ax}- or 9_{eq}-hydroxy (**6** and **11**–**13** with **10** as an exception), oxime (**14**), *O*- or *N*-methyl (**18** and **19**) or 13-hydroxy (**21**) substituents plus the non-ester ryanoid (**23**).

TABLE 2
Topical Toxicity of Ryanoids to House Flies Alone and with Piperonyl Butoxide (PBO)

		LD_{50} ($\mu g\ g^{-1}$)	
No.	*Compound*	*No PBO*	*With PBO*
Ryania active ingredients			
1	Ryanodine	290[a]	50
2	Didehydroryanodine	90	12
Modifications of cyclohexane substituents			
3	10-Deoxy-**2**	23	0·41
4	10-Oxo-**1**		2·5
7	21-*nor*-10-Deoxy		3·0
8	Methoxime-**1**		13
4,6-Cyclic boron derivatives			
16	Methylboronate-**2**	180[a]	14
17	Borate-**2**		250[a]

[a] Extrapolated from 25–45% mortality at 150 $\mu g\ g^{-1}$.

3.3 Topical toxicity of ryanoids (Table 2)

Ryanoids **1** and **2** are moderately active applied topically after PBO to retard metabolic detoxification. Compound **2** is more toxic than **1** topically but not when injected. There is little potency change in topical activity on conversion to the methoxime (**8**) or methylboronate (**16**) whereas the borate (**17**) is much less active. The borate (**17**) is more acidic and hydrophilic than the methylboronate (**16**), which may impede transport across the cuticle. Significantly, **1** and **2** become much more effective on removing the 10-hydroxyl substituent (**3** and **7**) or oxidizing it to a ketone (**4**). The synergized topical activity increase for **3** versus **2** of 29-fold is possibly associated with greater lipophilicity on removal of the hydroxyl substituent and this enhanced potency is also evident without PBO. Compound **3** approximates the toxicity to house flies of *S*-bioallethrin (0·32 $\mu g\ g^{-1}$ with PBO and 14 $\mu g\ g^{-1}$ without synergist).[13]

3.4 Toxicity to mice

Mouse ip LD_{50} values are 0·15 and 0·45 mg kg^{-1} for compound **2** and its 10-deoxy derivative (**3**), respectively, in agreement with their three-fold difference in potency at the *ryr* (Table 1). Thus, the increase in insecticidal activity of **3** versus **2** is not coupled to an increase in mammalian toxicity.

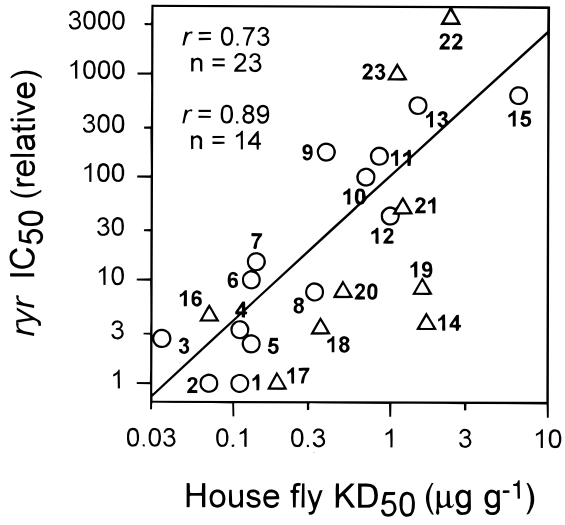

Fig. 4. Relation for ryanoids between injected KD_{50} for house flies pretreated with piperonyl butoxide and relative IC_{50} at the rabbit muscle Ca^{2+} release channel/ryanodine receptor (*ryr*). Data from Table 1. Correlation coefficients are for all compounds ($n = 23$) or only those with cyclohexyl modifications (except **14**) ($n = 14$, correlation line illustrated).

3.5 Relationship between injected KD_{50} and *ryr* IC_{50} (Table 1, Fig. 4)

The potency of most of the compounds is known for inhibition of the Ca^{2+}-release channel/*ryr* of rabbit skeletal muscle[2,10,11] and it is therefore of interest to determine the degree of relationship, if any, with house fly KD_{50} activity. There is a general correlation between receptor potency and KD_{50}, despite the species and system differences, suggesting that the target of the test compounds is the Ca^{2+} release channel. The correlation coefficient increases from 0·73 for all 23 compounds to 0·89 for 14 of the 15 compounds with modified cyclohexyl substituents (excluding oxime **14**). This correlation indicates a lack of target site selectivity, i.e. insect versus mammalian *ryr*. However, the non-ester ryanoids **22** and **23** do not fit the correlation line and are therefore exceptions to this relationship. Selective toxicity of the ester and non-ester ryanoids may result from their relative affinities for the same type of target (e.g. *ryr* of house fly and rabbit[5]) or from acting on fundamentally different targets, e.g. Ca^{2+} and K^{+} channels for the ester and non-ester ryanoids, respectively.[6,7]

4 CONCLUSIONS

These studies, an early stage in structure simplification and optimization, establish that the insecticidal activity of the natural ryanoids can be improved by structural modifications. Thus, ryanoids continue to serve as challenging prototypes for a potential new generation of insecticides.

5 ACKNOWLEDGMENTS

The project described was supported by Grant PO1 ES00049 from the National Institute of Environmental Health Sciences, NIH. We thank Gary Quistad, Susan Sparks and Motohiro Tomizawa of this laboratory for advice and assistance.

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